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# Gut microbiota composition is associated with narcolepsy type 1

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## Abstract

### Objective

To test the hypothesis that narcolepsy type 1 (NT1) is related to the gut microbiota, we compared the microbiota bacterial communities of patients with NT1 and control subjects.

### Methods

Thirty-five patients with NT1 (51.43% women, mean age  $38.29 \pm 19.98$  years) and 41 controls (57.14% women, mean age  $36.14 \pm 12.68$  years) were included. Stool samples were collected, and the fecal microbiota bacterial communities were compared between patients and controls using the well-standardized 16S rRNA gene amplicon sequencing approach. We studied alpha and beta diversity and differential abundance analysis between patients and controls, and between subgroups of patients with NT1.

### Results

We found no between-group differences for alpha diversity, but we discovered in NT1 a link with NT1 disease duration. We highlighted differences in the global bacterial community structure as assessed by beta diversity metrics even after adjustments for potential confounders as body mass index (BMI), often increased in NT1. Our results revealed differential abundance of several operational taxonomic units within Bacteroidetes, *Bacteroides*, and *Flavonifractor* between patients and controls, but not after adjusting for BMI.

### Conclusion

We provide evidence of gut microbial community structure alterations in NT1. However, further larger and longitudinal multiomics studies are required to replicate and elucidate the relationship between the gut microbiota, immunity dysregulation and NT1.

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## Glossary

**BMI** = body mass index; **EDS** = excessive daytime sleepiness; **IQR** = interquartile range; **NT1** = Narcolepsy type 1; **MSLT** = Multiple Sleep Latency Test; **ORX** = orexin; **OTU** = operational taxonomic unit; **SOREMP** = sleep-onset REM periods.

Narcolepsy type 1 (NT1) is an orphan disorder characterized by excessive daytime sleepiness (EDS), cataplexy, hypnagogic hallucinations, sleep paralysis and disturbed nighttime sleep.<sup>1–3</sup> This sleep disease is associated with psychiatric, cardiovascular, autonomic and metabolic disorders.<sup>3</sup> NT1 is also called orexin (ORX)/hypocretin deficiency syndrome, as it is caused by the irreversible loss of ORX neurons in the lateral hypothalamus.<sup>4,5</sup> Patients subsequently need lifelong management with symptomatic medications.<sup>6,7</sup> The precise underlying process leading to this destruction remains unclear; however, several arguments strongly support an autoimmune origin,<sup>8</sup> with the tight association with immune-related genetic factors, mainly the HLA DQB1\*06:02 allele, the T-cell receptor alpha, and specific adaptive immune response directed to ORX neurons.<sup>9–11</sup> The rarity of familial cases and the low concordance rate between monozygotic twins highlight the key role of environmental factors in the etiology of NT1.<sup>6,12</sup> Well-designed environmental studies are rare in NT1 with low reproducibility, except for the recently discovered triggers H1N1 influenza infection and vaccination during the 2009 influenza pandemic.<sup>13,14</sup> An association with *Streptococcus pyogenes* infections was also reported in NT1, with high anti-streptolysin O titers when close to disease onset,<sup>8</sup> but it was not replicated in a recent study.<sup>15</sup> Although studies proving causality between environmental factors and NT1 are rare, it has been hypothesized that infections can trigger the development of the disease by molecular mimicry and bystander activation mechanisms.<sup>11</sup>

The gut microbiota is home to an estimated 100 trillion bacteria, archaea, fungi and other microbial eukaryotes, as well as viruses, with the total genome estimated to contain approximately 3 million genes, 100-fold more than the human genome.<sup>16,17</sup> These organisms have evolved to live in symbiosis with their human host. However, the composition of the gut microbiome has the potential to influence pathogenesis of many disorders including neuro(auto)immune diseases (e.g., MS and neuromyelitis optica spectrum) and neurodegenerative diseases (e.g., Parkinson's disease, Alzheimer's disease, Huntington's disease) through the microbiota-gut-brain axis, and more widely, obesity, diabetes, liver diseases and cancer.<sup>18,19</sup> A growing body of evidence demonstrates the complex interactions between the gut microbiota and the CNS.<sup>18–20</sup>

Recent advancements with application of high-throughput sequencing technology (e.g., 16S rRNA gene sequencing<sup>21</sup>) allowed to identify many of the unculturable microorganisms that constitute the human gut microbiota. Differences in microbiota diversity and relative abundance have been

reported in neuroimmune diseases.<sup>18–20</sup> However, whether gut microbiota dysbiosis is causal or a contributing factor, involved in onset and/or progression of the disease remains unclear for most of these disorders.<sup>18–20</sup>

Assessment of the intestinal microbiome in NT1 is a new field of investigation. The potential role for gut microbiota in the pathogenesis of NT1 remains an open question, as well as the possible, yet hypothetical, presence of a specific microbial signature. To explore this hypothesis, we compared the fecal microbiota bacterial communities of well-defined patients with NT1 and control subjects using a 16S rRNA gene amplicon sequencing approach.

## Methods

### Standard protocol approvals, registrations, and patient consents

This study was approved by the local ethics committees (Comité de Protection des Personnes, France: Constitution of a cohort and of a clinical, neurophysiologic and biological bank of rare hypersomnolence disorders). All adult participants and both parents for minors provided written informed consent prior to participation.

### Study population

Thirty-five patients with NT1 (51.43% women, mean age: 38.29 ± 19.98 years, 5 children >12 y.o.) diagnosed at the National Reference Center for Narcolepsy, Montpellier, France were recruited from March 2018 to February 2019. The diagnosis of NT1 was based on the third International Classification of Sleep Disorders (ICSD-3) criteria<sup>22</sup>: presence of EDS for at least 3 months, mean sleep latency ≤8 minutes on the Multiple Sleep Latency Test (MSLT), with at least 2 sleep-onset REM periods (SOREMPs), and typical cataplexy or low CSF levels of ORX-A (<110 pg/mL) when a lumbar puncture was performed. All patients were positive for HLA-DQB1\*06:02. CSF ORX-A level was determined in 29 (83%) patients in duplicate using the <sup>125</sup>I-radioimmunoassay (RIA) kit from Phoenix Pharmaceuticals, Inc., (Burlingame, CA) according to the manufacturer's recommendations. All had low levels of ORX-A. Patients were systematically evaluated for body mass index (BMI) (categorized as normal weight [<25 kg/m<sup>2</sup>], overweight [25–30 kg/m<sup>2</sup>], and obese [≥30 kg/m<sup>2</sup>], and according to BMI growth curves for children), age at disease onset and disease duration, and medication intake at time of the study. None of these patients had significant comorbid psychiatric or medical disorders.

We also recruited 41 controls (57.14% women, mean age: 36.14 ± 12.68 years, 4 children >12 y.o.) during the same period:

18 (44%) were healthy controls selected from the general population via advertisement and local association networks, and 23 (56%) had an initial EDS complaint, but none of them fulfilled criteria for a central hypersomnolence disorder (including narcolepsy). These 23 subjects had no objective sleepiness on the MSLT (mean sleep latency >8 minutes), no SOREMP, no abnormalities on nocturnal polysomnography, no complaint of long sleep time, no medication use, and no other significant psychiatric, neurologic, or medical disorders.

None of the participants (NT1 and C) were affected with comorbid neurologic inflammatory or immune pathologies. None of them were treated with antibiotics, immunotherapy, or corticosteroids at the time of the study.

### Stool sampling

Stool samples were collected for all participants in the sleep unit from March 2018 to February 2019 using DNA Stool Collection Tubes containing DNA Stabilizer from the PSP® Spin Stool DNA Plus Kit of STRATEC Molecular®, and subsequently frozen and stored at –80°C. Stool samples were shipped on dry ice to the DNA Sequencing and Genomics laboratory at the Institute of Biotechnology, University of Helsinki, Finland for 16S rRNA gene analysis.

### DNA extraction

DNA extraction of the samples was performed using the STRATEC Molecular® PSP Spin Stool DNA Plus Kit according to the manufacturer's instructions. Samples were randomized between extraction batches to minimize batch effects. Blank extractions were performed from DNA Stabilizer tubes with no addition of template as a way to assess the presence of possible contaminants.

### Library preparation and sequencing

Libraries were obtained by performing 2 steps of PCR. In the first step the V3-V4 regions of the 16S rRNA gene were amplified following a previously published protocol.<sup>23,24</sup> We used 2 technical replicates (25 µL reactions) per subject sample, and a mixture of the universal bacterial primers 341F1–4 (5' CCTACGGGNGGCWGCAG 3') and 785R1–4 (5' GACTACHVGGGTATCTAATCC 3') with partial TruSeq adapter sequences added to the 5' end.<sup>23</sup> PCR was done with the following conditions: initial denaturation at 98°C for 30 seconds, 12 cycles at 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 10 seconds, and a final extension at 72°C for 5 minutes. A clean-up step was performed to remove unused primers and nucleotides following a previously published protocol.<sup>24</sup> In the second PCR step the full-length P5 and P7 Illumina adapters containing 8 bp indexes (dual-index) were added. The used indexes were selected using BARCOSEL.<sup>25</sup> The two-step PCR and subsequent quantification, pooling, and purification were done as described previously.<sup>24</sup> The obtained PCR amplicon pool was checked using Fragment Analyzer (Advanced Analytical Technologies Inc., Ankeny, IA). PCR of the samples and the isolation controls were done in batches and every PCR batch included a blank control (no added DNA template for

amplification) to assess potential contamination. Finally, the PCR products were sequenced with Illumina MiSeq using the v3 600 cycle kit paired-end (325 bp + 285 bp). All the samples were sequenced in a single run to avoid batch effects. The raw sequence data contained a total of 24,660,868 paired-end reads.

### Sequencing data

Primers were removed from the reads using cutadapt (version 2.3)<sup>26</sup> with default parameters, an error rate of 10%, minimum length of overlap between read and adapter set as 3 bp, with command line provided in supplementary data (Supplementary methods, [links.lww.com/NXI/A322](https://links.lww.com/NXI/A322)). The reads were paired, quality trimmed, taxonomically classified, and clustered into operational taxonomic units (OTUs) following Mothur's Standard Operating Procedure (SOP) for MiSeq (Mothur 1.42.1; SOP version last updated on April 4, 2018).<sup>27</sup> We inspected the extraction and PCR blanks, which overall had very low amounts of sequence reads, and since these did not suggest any overall problems with contamination, we removed these samples before downstream analysis. The final sequence data set (without the blanks) consisted of 7,460,575 good quality reads.

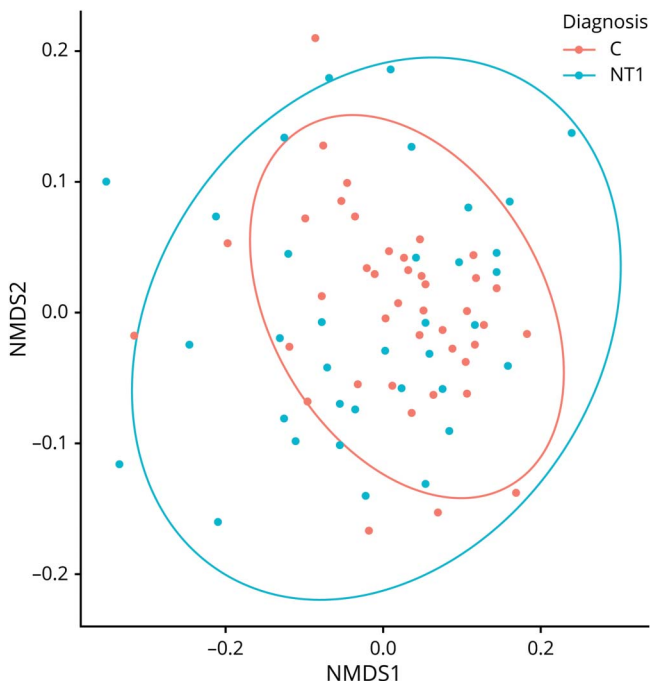
### Statistical analysis

All statistical comparisons and data visualization were performed with the R statistical programming language (v. 3.6.1).<sup>28</sup> Quantitative variables are presented as mean and SD, or median with interquartile range (IQR). All *p*-values are double-tailed, with statistical significance accepted at an alpha of 0.05 in alpha and beta diversity, and at an alpha of 0.1 in differential abundance analysis.

Non-rarefied data without singletons were used for calculating alpha diversity indices (observed richness, Shannon index and Inverse Simpson). These 3 different indices were compared statistically between groups using the Wilcoxon rank sum test, and Spearman's rank correlation test. Beta diversity, based on Bray-Curtis dissimilarity, was performed with rarefied data (37,008 sequences per sample). Beta diversity was visualized with Non-Metric Multidimensional Scaling and compared statistically with *adonis2* (an implementation of PERMANOVA) from the *vegan* package.<sup>29</sup>

Differential abundance analyses were done with DESeq2,<sup>30</sup> which uses Generalized Linear Models with a negative binomial distribution, with Benjamini-Hochberg method for multiple comparisons correction. A complementary differential abundance analysis was done with ALDEx2,<sup>31</sup> which uses a Dirichlet-multinomial model to infer abundance from counts. Predicted metagenomic functional potential of the microbial community was obtained by using PICRUSt2 (v. 2.3.0 beta) (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States),<sup>32,33</sup> and following the tutorial available on GitHub. Visualization and statistical comparison of differential abundances in predicted metagenomic functional potential was performed using STAMP (v. 2.1.3) (statistical analysis of taxonomic and functional profiles)<sup>34</sup> and following PICRUSt

**Figure 1** NMDS ordination plot of the control (C) and narcolepsy (NT1) samples



Ordination based on Bray-Curtis dissimilarity calculated with genus-level data, randomly rarefied to 37,008 sequences per sample. Ellipses indicate 95% confidence intervals. Each point represents one sample. The closer the points are to one another, the more similar the microbiome compositions of the samples are. NMDS = Non-Metric Multidimensional Scaling; NT1 = Narcolepsy type 1.

visualization and statistics in STAMP provided in PICRUSt tutorial. EC (Enzyme Classification) and KEGG (Kyoto Encyclopedia of Genes and Genomes) Ortholog pathway predictions were analyzed after correction for multiple comparisons (Benjamini-Hochberg FDR).

### Data availability

Anonymized data not provided in the article because of space limitations will be shared by request from any qualified investigator.

## Results

### Characteristics of the study population

At the time of the study, the duration of NT1 was  $221.5 \pm 198.8$  months. Thirteen patients (37%) had a normal BMI, 12 (34%) were overweight and 10 (29%) were obese. At time of study entry, 26 patients were drug-free while 9 were treated for narcolepsy: modafinil ( $n = 4$ ), methylphenidate ( $n = 5$ ) alone or in combination with pitolisant ( $n = 1$ ), sodium oxybate ( $n = 1$ ), venlafaxine ( $n = 3$ ) and clomipramine ( $n = 1$ ).

Healthy controls and the control population with a complaint of EDS did not significantly differ for age, gender and BMI, and neither in alpha nor beta diversity. Thus, both control

populations were combined to form a single larger control group. Initial analysis revealed that with respect to microbiome compositional data, one control subject was a major outlier for unknown reasons. We chose to not include this control subject in subsequent analyses. We found no between-group differences for age and gender between patients with NT1 and controls. However, BMI was higher in patients than controls (median [IQR]: 26.2 [23.3–30.3] vs 23.1 [20.6–24.2],  $p < 0.001$ ).

### Alpha diversity

Alpha diversity defined as microbial community richness and evenness did not differ between patients with NT1 and controls as assessed with the Shannon (Wilcoxon rank sum test:  $p = 0.07814$ ,  $W = 887$ ) and Inverse Simpson (Wilcoxon rank sum test:  $p = 0.06801$ ,  $W = 893$ ) indices, nor with observed richness (Wilcoxon rank sum test:  $p = 0.1281$ ,  $W = 864$ ). Alpha diversity was also investigated as function of age, gender and BMI, with no significant effects found (Supplementary table 1, [links.lww.com/NXI/A321](https://links.lww.com/NXI/A321)).

We further analyzed in patients with NT1 the association between alpha diversity and demographic and clinical characteristics such as gender, age, disease duration, BMI, and medication (Supplementary table 2, [links.lww.com/NXI/A321](https://links.lww.com/NXI/A321)). We found no associations except between the Shannon index (i.e., a commonly used index to characterize diversity in terms of richness and evenness) and disease duration (Spearman's rank correlation test:  $p = 0.038$ ,  $r = 0.352$ ).

### Beta diversity

Beta diversity quantifies the community composition dissimilarity between samples and showed significant differences between patients and controls using Bray-Curtis dissimilarity in univariable PERMANOVA models ( $p = 0.026$ ,  $R^2 = 0.023$ ) (figure 1). We also found trends for dissimilarity for gender ( $p = 0.0549$ ,  $R^2 = 0.02$ ) and BMI ( $p = 0.082$ ,  $R^2 = 0.019$ ), but not for age, disease duration and medication. The difference between patients and controls remained significant after adjustment for gender and BMI in a multivariable model (Diagnosis:  $p = 0.027$ ,  $R^2 = 0.023$ ; Gender:  $p = 0.032$ ,  $R^2 = 0.022$ ; BMI:  $p = 0.055$ ,  $R^2 = 0.02$ ).

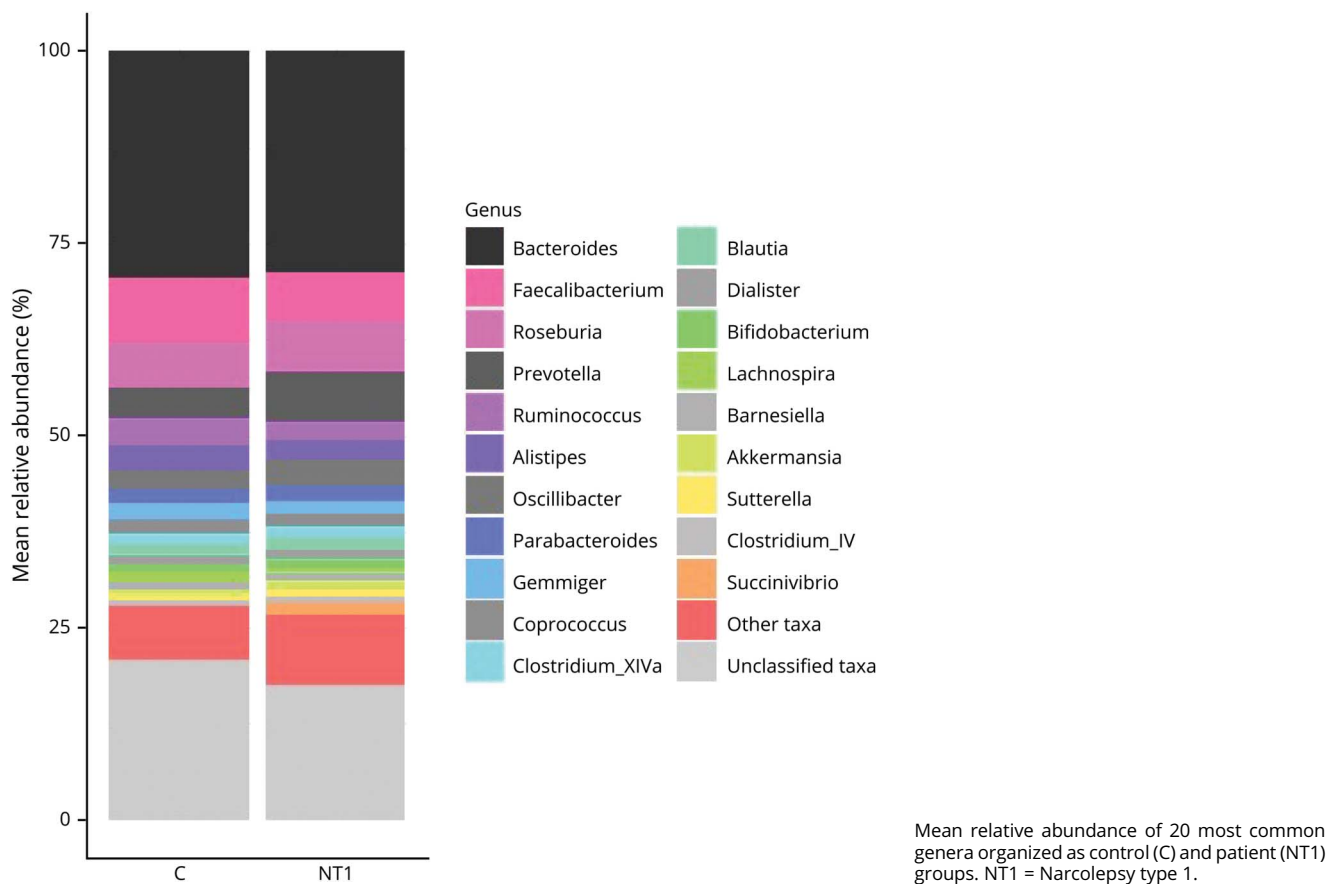
We further analyzed the Beta diversity in patients with NT1 in univariable models as a function of gender, age, disease duration, BMI, and medication, but found no significant associations.

### Differential microbial abundance analyses between patients with NT1 and controls

The data set was composed of 7,243 non-singleton OTUs distributed into 180 genera, 73 families, 36 orders, 22 classes and 12 phyla. The 20 most common and the 5 most abundant genera (e.g., *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Prevotella*, and *Ruminococcus*) in patients and controls are shown in figure 2. We analyzed the differential abundance at different taxonomic levels, namely OTU, genus, family, and phylum levels with DESeq2. For every taxon level, we identified and plotted the normalized counts and the relative abundances of



**Figure 2** The most 20 common genera between patients with NT1 and controls



the significant differentially abundant taxa if any, the 20 most common taxa, as well as the 20 taxa with the lowest corrected *p*-value for multiple comparisons. We found several taxa with differential abundance between patients with NT1 and controls (table 1 and figure 3). *Bacteroides* Otu00012 and *Bacteria unclassified* Otu00078 were decreased in NT1 compared to controls, while *Flavonifractor* Otu00099 and *Bacteroidetes unclassified* Otu00102 were increased in NT1 compared to controls. After adjusting for gender, 3 among the 4 taxa remained significant between groups: *Bacteroides* Otu00012, *Bacteria unclassified* Otu00078 and *Flavonifractor* Otu00099. However, after correction for BMI alone, and then gender and BMI in the same model, no taxa remained statistically significant for differential abundance between groups. Few other between-group differences were identified at OTU, genus, family and phylum taxonomic levels. However, a detailed review of the normalized counts plots and the relative abundance plots showed several outliers in both groups and thereafter these taxa could no longer be considered as different between the groups.

A complementary differential abundance analysis was done with ALDEx2 at all taxonomic levels; however we found no

significant differences between groups in any of the comparisons performed.

### Relative microbial abundance analyses according to demographic and clinical characteristics in patients with NT1

We analyzed the differential abundance analysis at genus level within patients with NT1 according to age (in children compared to adults), disease duration (population divided as the median of disease duration, adjusted only for age), BMI (population divided into obese, non-obese), and medication (in untreated and then in treated patients). Among these subgroups, DESeq2 identified the following taxa as differentially abundant (Age: *Acidaminococcus*, *Allisonella*, *Alloprevotella*, *Catenibacterium*, *Holdemanella*, *Klebsiella*, *Prevotella*; disease duration: *Alloprevotella*, *Butyrivibrio*; BMI: *Alloprevotella*, *Clostridium IV*, *Coprobacillus*, *Intestinimonas*, *Mitsuokella*; medication: *Faecalicoccus*, *Klebsiella*, *Parasutterella*, *Veillonella*). However, the sample size of the different subgroups was small, and the review of the normalized counts and the relative abundance plots showing the presence of outliers suggested that the significant differences were rather statistical noise than true between-group differences.

**Table 1** Significantly differentially abundant taxa in patients with NT1 and controls

Groups	Variable	Taxon level	Taxon designation	Normalized mean abundance	Log 2 fold change	Adjusted p Value
All C and all NT1	Diagnosis	OTU	<i>Bacteroides</i> Otu00012	1,058.259	+13.999 in C	8.66e-16
All C and all NT1	Diagnosis	OTU	<i>Bacteria unclassified</i> Otu00078	109.098	+8.144 in C	3.18e-04
All C and all NT1	Diagnosis	OTU	<i>Flavonifractor</i> Otu00099	91.947	+1.697 in NT1	1.64e-02
All C and all NT1	Diagnosis	OTU	<i>Bacteroidetes unclassified</i> Otu00102	62.685	+10.048 in NT1	2.10e-02
All C and all NT1	Diagnosis corrected for gender	OTU	<i>Bacteroides</i> Otu00012	1,058.259	+13.94 in C	3.78e-16
All C and all NT1	Diagnosis corrected for gender	OTU	<i>Bacteria unclassified</i> Otu00078	173.435010	+8.01 in C	1.66e-04
All C and all NT1	Diagnosis corrected for gender	OTU	<i>Flavonifractor</i> Otu00099	91.946991	+1.6376 in NT1	5.10e-03

Abbreviations: C = controls; NT1 = narcolepsy type 1; OTU = operational taxonomic unit.

Variation of the significantly differentially abundant taxa without correction and after correction for gender. Log2FoldChange: estimation of the effect size (as a fold change) in log 2 scale and orientation of the variation; it represents how much does the abundance of the taxum of interest changes between groups (C and NT1). It also gives the orientation of this change.

## Predicted metabolic pathways

PICRUSt2's predictions of microbial metabolic pathway abundances were used to infer potential differences between patients with NT1 and controls. We found no significant differences in predicted metabolic function patterns between patients with NT1 and controls.

## Discussion

Here, we report an assessment of gut microbiota diversities and relative abundances in a well-characterized population of patients with NT1 compared with control subjects. We found no between-group differences for alpha diversity that quantifies both taxonomic richness and evenness, except for the Shannon index that correlated with NT1 disease duration. This result suggests that richness and evenness of microbial species may differ as a function of disease evolution in patients with NT1. We also reported a significant difference regarding the global bacterial community structure in NT1 vs controls, as assessed by beta diversity metrics. These results highlighted that the microbial community differs between patients with NT1 and controls, even after adjustment for BMI. We further explored with the DESeq2 analyses whether the community differences between groups are driven by changes in the abundance of certain taxa. Few microbial species were differentially abundant between patients and controls with one OTU among *Bacteroides* and one OTU among unclassified bacteria, which were decreased in patients, whereas an OTU among unclassified *Bacteroidetes* and one OTU among *Flavonifractor* were increased in patients with NT1 compared with controls. After adjustment for BMI, these differences did not remain significant and thus may not be associated with NT1 per se. Importantly, metabolic disturbances such

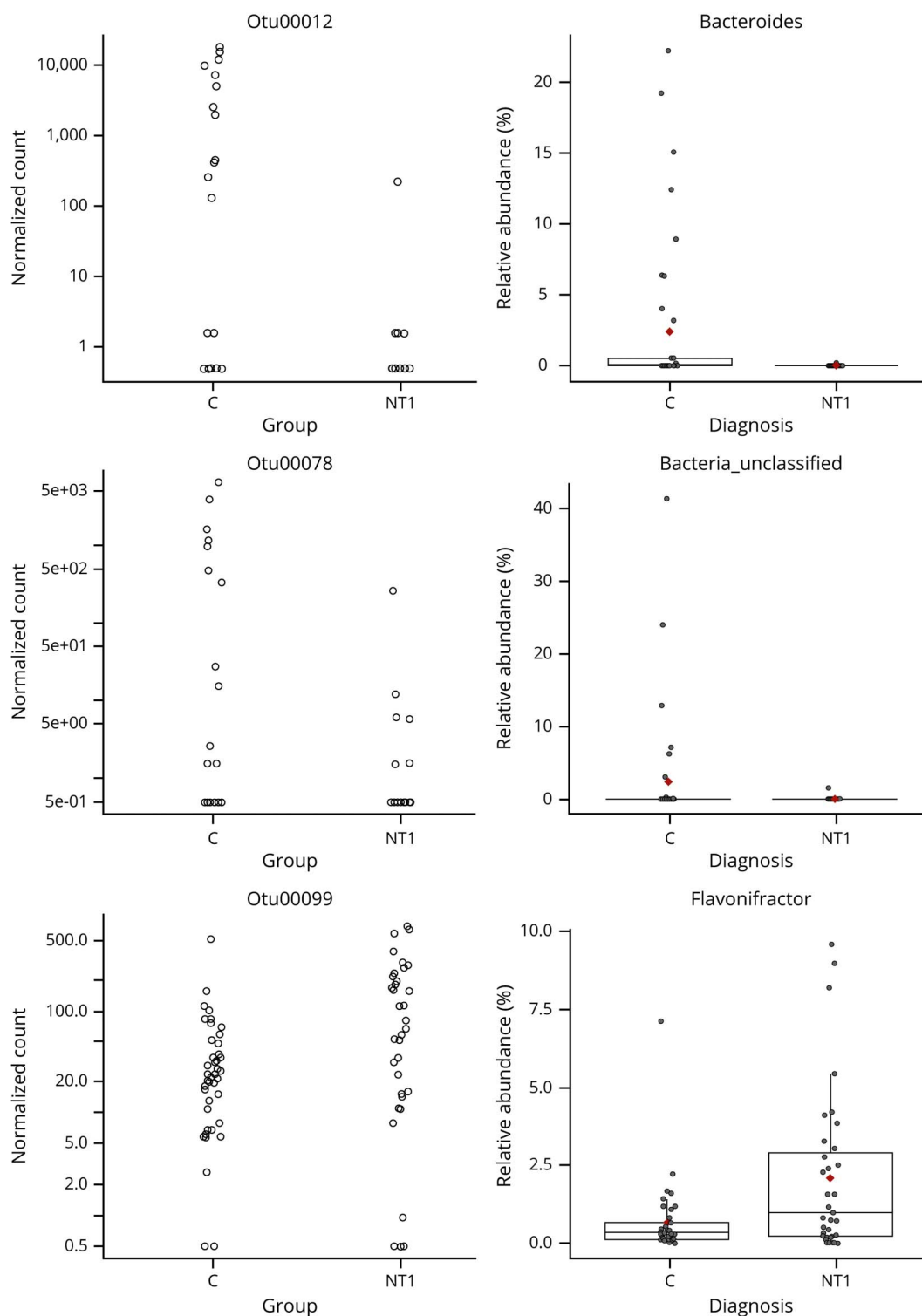
as overweight/obesity are frequently associated with NT1: one-third of adults with NT1 are obese, and 50% of children, with a rapid weight increase close to disease onset.<sup>3,35,36</sup>

Several studies have attempted to define what constitutes an “obese” vs a “healthy” gut microbiota.<sup>37,38</sup> Generally, microbial diversity is an essential component to host health, and obese individuals have a lower bacterial diversity and decreased fecal microbial richness.<sup>39</sup> The question whether changes in beta diversity and subtle differential community abundance between patients with NT1 and controls were directly related to the disease per se or to confounders such as BMI needs to be further explored.

Our global results may argue for a shift of the whole community structure in patients with NT1 compared with controls. Using the DESeq2 analyses, we found that the community differences between groups may relate to changes in abundance of few taxa; however, a complementary abundance analysis performed with the ALDEx2 at all taxon levels, found no between-group statistically significant results. ALDEx2 is a very conservative method, and it is not uncommon that results detected by DESeq2 or other methods are not reproduced by ALDEx2.<sup>23</sup> The analysis of predicted metabolic pathways did not reveal functional differences between groups that would reflect the observed compositional differences. Finally, we did not find any association between microbial community structure and demographic and clinical characteristics in patients with NT1.

Although preliminary, the gut microbial diversity alteration we found here in NT1 reinforces the hypothesis of the important role of the environment in NT1 development and pathogenesis. It is now widely accepted by the scientific community that the

**Figure 3** Normalized counts and relative abundance plots between patients with NT1 and controls



Visualization of differential abundance with plot of normalized counts and relative abundances of bacterial OTUs in the control (C) and narcolepsy (NT1) groups. Lower and upper hinge of the box: 1st and 3rd quartile; line: median; red diamond: mean. Three OTUs are represented: *Bacteroides* Otu00012, *Bacteria unclassified* Otu00078, and *Flavonifractor* Otu00099—the significantly differentially abundant OTUs (DESeq2  $p < 0.1$ ) between control and NT1 groups after adjusting for gender. NT1 = Narcolepsy type 1; OTU = operational taxonomic unit.

immune system is involved in the death of ORX neurons.<sup>3,40</sup> Complex interactions have been established between the gut microbiota and the CNS, and gut-brain interactions may occur

through immune-mediated inflammatory pathways.<sup>18,19</sup> The gut microbiota plays a key role in maintaining the integrity of the intestinal epithelium and the mucosal barrier.<sup>39</sup> Several other



roles of the gut microbiota were highlighted with modulation of CNS inflammation, regulatory cells from both T and B cell lineages, and changes in cytokine activities and microglia activation, all potential players involved in the pathogenesis of NT1.<sup>18,19,41–43</sup> Hence, subtle changes in the microbiome may modulate risk of NT1 in genetically predisposed individuals (carrying the HLA DQB1\*06:02 allele) as already reported in several neuro(auto)immune disorders such as MS.<sup>18,44</sup>

At this point, it remains unclear whether the changes in the gut microbiota are robust in NT1 and whether these changes, if confirmed, are involved in disease causation or are rather a consequence of the disease. Regarding the latter, it also cannot be ruled out that latent identified (such as BMI) or unidentified clinical confounders are involved, meaning that observed beta diversity and taxon abundance differences may not even be a consequence of the disease state itself. In addition, how microbiota affect changes in (auto)immunity related to NT1, symptom burden, disease progression, and drug treatment responses remains to be further established. Whether the modulation of the microbiome can lead to either exacerbation or improvement of symptoms in NT1, especially close to disease onset, remains also to be investigated.

This study has several strengths and limitations. We included well-characterized patients with NT1 comparable with control subjects about gender and age, with recruitment of subjects within the same geographical region and from the same period. None of the participants had comorbid immune diseases, an expected condition in NT1; this disorder having a unique pathophysiology.<sup>45</sup> Experimental design with standardized collection and storage; molecular analysis technology and methods for data analysis were performed according to the best practice methods, with strong precautions to minimize batch effects and contamination.<sup>46,47</sup> The main limitation of our study is the small sample size. However, NT1 affects only 1 in 2,000 people worldwide, so the rarity of this sleep disorder justifies the present report of the first small-scale exploratory study of the gut microbiota in NT1. Unfortunately, owing to heavily tailed zero-inflated distributions of bacterial abundances and the multivariate nature of the data, power calculations for microbiome studies are challenging, and no consensus exist on how they should be conducted. The question of whether the difference in beta diversity and subtle changes in relative abundance of microbial taxa between narcolepsy and control samples reflects an underlying diversity or sampling bias remains to be confirmed. Patients with NT1 had a higher BMI than control subjects, which may have introduced bias into the analysis despite our model adjustments. In addition, our study lacks assessments of lifestyle, diet as well as gastrointestinal discomfort in our subjects, and questionnaires on gut and on depressive symptoms, which would have further improved confounder adjustment. Indeed, a recent study reported a large spectrum of clinical autonomic dysfunction in NT1 with impairment in the gastrointestinal area that may modify the gut microbial composition.<sup>48</sup> Moreover, most of the patients were recruited long after disease onset, but long delays between symptoms onset and diagnosis remain unfortunately

the rule for this rare disease.<sup>49</sup> Therefore, possible transitory microbiota alterations, more pronounced during the autoimmune processes at disease initiation, may have become undetectable during the follow-up period. Owing to the lack of power in the analysis of subgroups of patients based on clinical characteristics, we cannot at this stage discern the effects of endophenotypes, comorbidities, and of medications taken by patients with NT1 on abundances in the microbial community. Finally, our results cannot be considered as definite identification of specific gut microbial community structure alterations in NT1. We need larger sample sizes in the future to obtain more reliable estimates, and different study designs with longitudinal studies and randomized controlled trials with multiple time points of the same subjects to elucidate potential causal relationships and to find out if targeting the microbiome can yield novel therapeutic strategies.

To conclude, we provide evidence for differences in gut microbial communities in NT1. However, our preliminary results cannot be considered a definite identification of specific microbiome-based biomarkers in narcolepsy. Further larger, and preferably longitudinal, multiomics studies are required to replicate our findings, to elucidate the relationship between the gut microbiota and NT1, and to determine the role of the microbiome in the development of the disease, if any.

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## Appendix Authors

Name	Location	Contribution
<b>Alexandre Lecomte, MSc</b>	University of Helsinki, Finland	Data acquisition, drafting/revising the manuscript, study concept or design, analysis or interpretation of data, statistical analysis, study supervision, and contribution of vital reagents/tools/patients
<b>Lucie Barateau, MD</b>	University of Montpellier, France	Data acquisition, drafting/revising the manuscript, and analysis or interpretation of data
<b>Pedro Pereira, PhD</b>	University of Helsinki, Finland	Drafting/revising the manuscript, study concept or design, analysis or interpretation of data, statistical analysis, and study supervision
<b>Lars Paulin, MSc</b>	University of Helsinki, Finland	Data acquisition and drafting/revising the manuscript
<b>Petri Auvinen, PhD</b>	University of Helsinki, Finland	Data acquisition, drafting/revising the manuscript, study concept or design, analysis or interpretation of data, and study supervision
<b>Filip Scheperjans, MD</b>	University of Helsinki, Finland	Drafting/revising the manuscript, study concept or design, analysis or interpretation of data, and study supervision
<b>Yves Dauvilliers, MD</b>	University of Montpellier, France	Data acquisition, drafting/revising the manuscript, study concept or design, analysis or interpretation of data, study supervision, and obtain funding

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